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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Hauptmann *et al.*	Art Unit: 1812
Appl. No.: 08/249,671	Examiner: Fitzgerald, D.
Filed: May 26, 1994	Atty Docket: 0652.1350000/RWE/LLK
For: **Process for Preparing and Purifying Alpha-Interferon**	

considered
DF 7/10/97

Declaration Under 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

The undersigned, Rudolf Hauptmann, declares and states that:

1. I received a Dr.rer.nat. (equivalent to a Ph.D.) at the University of Vienna, located in Vienna, Austria, in 1976.

2. I am a coinventor of the above-captioned patent application.

3. Since 1982, I have been employed by the Ernst Boehringer Institut für Arzneimittelforschung of Bender & Co. GmbH in Vienna, Austria. A copy of my curriculum vitae is attached hereto as Exhibit A.

4. I have read and I am familiar with the prosecution of this application, including the Office Action of May 16, 1996, wherein the Examiner rejected claims 1-3 and 17-19 as obvious over

Miyake *et al.* in view of Chang *et al.*, and further in view of Vandlen *et al.*, Capon *et al.*, and Baxter *et al.*

5. Table 1 of Chang *et al.* shows that the amount of human growth hormone (hGH) obtained using a vector comprising a human hGH cDNA ligated to a sequence encoding the STII signal sequence (STII/hGH), under the control of a trp promoter, is twice as high (1 gram/50 OD/l) as the amount of hGH obtained using a vector comprising STII/hGH under the control of a phoA promoter (0.5 gram/OD/l).

6. In contrast, we have unexpectedly found that the IFN-α product yield is three times higher where IFN-α is expressed from the vector construct recited in the claims comprising IFN-α cDNA ligated to a sequence encoding the STII signal sequence (STII/IFNα) under the control of a phoA promoter (0.1-0.2 mg/OD/l) as compared to a STII/IFN-α construct under the control of a trp promoter (0.06 mg/OD/l) (see Exhibit B, attached hereto, which details the construction of the relevant plasmids, the experiments performed, including controls, and the results obtained; further explanation regarding the details of STII/IFN-α expression may be found in Voss *et al.*, *Biochem. J. 298*: 719-725 (1994) (Exhibit C)). Therefore, while Chang *et al.* would have suggested to one of ordinary skill in the art that the best yield of a mammalian protein could be obtained by linking the gene of interest to a STII leader sequence and expressing this construct from a trp promoter, we have unexpectedly discovered that much better product yields of IFN-α may be obtained by expressing a STII/IFN-α from a phoA promoter.

7. Therefore, it would not have been obvious to one of ordinary skill in the art at the time the invention was made to construct an expression vector for IFN-α according to Miyake *et al.*,

replacing the phoA signal-peptide encoding sequence employed by Miyake *et al.* with the STII signal sequence used by Chang *et al.* Rather, if one of ordinary skill in the art were to assume that the findings of Chang *et al.* regarding hGH expression would be relevant to the expression of IFN-α, the logical construct to make would have been a STII/IFN-α fusion under the control of a trp promoter.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Dec. 12th, 1996
Date

Rudolf Hauptmann
Rudolf Hauptmann

135.DEC

Exhibit A

CURRICULUM VITAE of Rudolf Hauptmann

1950	Born March 17 at Vienna / Austria.
1968	"Matura mit Auszeichnung (cum laudae) at the Bundesrealschule Wien 3., Radetzkystraße 2.
1968-1973	Study of Chemistry at the University of Vienna.
1973-1976	Thesis at the Institute of Biochemistry.
1976	Dr.rer.nat. (Ph.D.) at the University of Vienna.
1976-1977	"Assistent" at the Institute of Biochemistry.
1977-1980	"Assistent" at the Institute of Molecular Biology at the University of Vienna.
1980-1982	Post doctoral study at the University of Leicester, Leicester, UK, about cloning and sequencing of Influenza and Polio viruses.
Since 1982	Leader of the Molecular Biology laboratory I at the "Ernst Boehringer Institut für Arzneimittelforschung" of Bender&Co GmbH, Vienna / Austria.

Projects:

- Cloning, sequencing and bacterial expression of human and animal interferons (Interferon-α and interferon-ω).
- Bacterial expression of human Fce Receptor - soluble fragment
- Cloning, sequencing and bacterial expression of human vascular anticoagulant protein.
- Cloning and bacterial expression of the human TNF(Tumor Necrosis Factor)-binding protein.
- Development of GEMS (Gene expression modulation system) tester cell lines concerning the expression of cholesterol ester transfer protein (CETP) and apolipoprotein AI
- Oligonucleotide synthesis as a service for the individual molecular biology laboratories of the Ernst Boehringer Institut für Arzneimittelforschung (since 1985).
- DNA sequencing using fluorescence in combination with an automated sequencer as a service for individual molecular

biology laboratories of the Ernst Boehringer Institut für Arzneimittelforschung (since 1989).

- Molecular Biology part of TNF-alfa EG dossier (Sequencing, restriction mapping, copy number)
- EMAPII, an anti-angiogenic cytokine (expression in E.coli, genomic characterization, project coordination)

Since 1994 Group Leader in the department FEM

Since 1996 Project coordinator - Neoangiogenesis

R.Hauptmann, list of publications:

- R.Hauptmann, A.P.Czernilofsky, H.O.Voorma, G.Stöffler and E.Kuechler: Biochem.Biophys.Res.Comm. 56 (1974), 331-337: "Identification of a protein at the ribosomal donor-site by affinity labeling"

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of the region of the genome encoding capsid protein VP1 of neurovirulent and attenuated type 3 polioviruses"

- J.W.Almond, A.J.Cann, P.D.Minor, P.Reeve, G.C.Schild, R.Hauptmann and G.Stanway Reviews of Infectious Diseases 6 Suppl.2 (1984), S487-S493: "Nucleotide sequence from neurovirulent and attenuated strains of type 3 poliovirus"

- R.Hauptmann and P.Swetly: Nucleic Acids Res. 13 (1985), 4739-4749: "A novel class of human type I interferons"

- R.Hauptmann, E.Ostermann, C.Pieler, W.Spevak and P.Swetly: The 1985 TNO-ISIR Meeting in the Interferon System (1985), 63: "A novel class of human type I interferons"

- R.Hauptmann: Wiener Klinische Wochenschrift 7/8 (1986), 158-160: "Escherichia coli in der Gentechnik"

- A.Himmler, R.Hauptmann, G.R.Adolf and P.Swetly: DNA 5 (1986), 345-356: "Molecular cloning and expression in Escherichia coli of equine type I interferons"

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- A.Himmler, R.Hauptmann, G.R.Adolf and P.Swetly: J.Interferon Res. 7 (1987), 173-183: "Structure and expression in Escherichia coli of canine interferon-α genes"

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- A.Himmler, I.Maurer-Fogy, M.Krönke, P.Scheurich, K.Pfizenmaier, M.Lantz, I.Olsson, R.Hauptmann, C.Stratowa and G.R.Adolf: DNA 9 (1990), 705-715: "Molecular cloning and expression of human rat tumor necrosis factor receptor chain (p60) and its soluble derivative, tumor necrosis factor-binding protein"

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- S. Mörwald and R.Hauptmann: Atherosclerosis 109 (1994), 140 (Poster abstract):"Isolation and characterization of a human cytidine deaminase cDNA (apolipoprotein B mRNA editing enzyme)"

- C.-G. Liu, C. Maercker, M.J.Castañon, R.Hauptmann and G.Wiche: Proc. Natl. Acad. Sci. USA 93 (1996), 4278-4283: "Human plectin: Organisation of the gene, sequence analysis, and chromosomal localization (8q24)"

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Exhibit B

Construction of expression vectors pDH10 (phoA promoter-STII-IFNα2c) and pDH11(trp promoter-STII-IFNα2c)

All cloning procedures were essentially performed following standard protocols ("Molecular cloning - a laboratory manual" Sambrook,J., Fritsch, E.F., & Maniatis T. (1989), Cold Spring Harbor Laboratory Press (1989).

pRH284/T: The promoter plasmid pRH284/T was generated in an analogous way as the promoter plasmid pRH281/5 (Case 12/069; DE-OS 38 10 474). A set of ligated oligonucleotides (phoA1-phoA10) was ligated between the EcoRI and ClaI sites of pAT153:

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:->phoA1
AATTGGAGATTATCGTCACTGCAATGCTTCGCAATATGGCGCAAAATGAC
    CCTCTAATAGCAGTGACGTTACGAAGCGTTATACCGCGTTTTACTG

          :->phoA3
CAACAGCGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCC
GTTGTCGCCAACTAACTAGTCCATCTCCCCCGCGACATGCTCCATTTCGG
         phoA2<-:

                        :->phoA5
CGATGCCAGCATTCCTGACGACGATACGGAGCTGCTGCGCGATTACGTAA
GCTACGGTCGTAAGGACTGCTGCTATGCCTCGACGACGCGCTAATGCATT
                       phoA4<-:

                                  :->phoA7
AGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTAATCTTTTCAACAGC
TCTTCAATAACTTCGTAGGAGCAGTCATTTTTCAATTAGAAAAGTTGTCG
                                 phoA6<-:

                                            :->phoA9
TGTCATAAAGTTGTCACGGCCGAGACTTATAGTCGCTTTGTTTTTATTTT
ACAGTATTTCAACAGTGCCGGCTCTGAATATCAGCGAAACAAAAATAAAA
                                          phoA8<-:

TTAATGTATTTGCTCGAGAGGTTGAGGTGATTTTATGAGCTCGAATTCATC
```

AATTACATAAACGAGCTCTCCAACTCCACTAAAATACTCGAGCTTAAGTAGCT

phoA10<-:

The resulting plasmid (pRH284) was then modified in the analogous way as pRH 281/5 by replacing the HindIII-SalI part of pRH284 with the oligonucleotide pair EBI-456/EBI-459, thereby introducing the transcription terminator of phoA (H.Shuttleworth et al, Nucl.Acids Res. 14 (1986), 8689; C.N.Chang et al., Gene 44 (1986), 121-125).

:->EBI-456

AGCTTGGATCCGTCGACCGCGCCCGGCAGTGAATTTTCGCTGCCGGGTGG

ACCTAGGCAGCTGGCGCGGGCCGTCACTTAAAAGCGACGGCCCACC

TTTTTTTGCTGC

AAAAAAACGACGAGCT

EBI-459<-:

The resulting plasmid was named pRH 284/T.

STII-IFNα2c: The construction of the expression cassette containing the phosphatase promotor, the STII leader and the human IFN-a2c was performed by SOE-PCR (Splicing by overlap extension, Ho et al., 1989). The IFN-α2c sequence was PCR-amplified from the HindIII-linearized bacterial expression construct pER21/1 (Dworkin-Rastl, E., Swetly, P. & Dworkin, M.B. (1983) Gene 21, 237-248) using the 5' primer (ATGCCTATGCATGTGATCTGCCTCAAA-CCCACAGC) and the 3' primer (GACTTCAGAAGCTTCTGCAGTTA-CGATCGTTATCATTCCTTACTTCTTAAACTTTC, Hind III site underlined). The phosphatase promotor (Chang et al., 1986, Shuttleworth et al., 1986) and the STII leader (Lee, C.H., Mosely, S.L., Moon, H.W., Whipp, S.C., Gyles, C.L. & So, M. (1983) Infection & Immunity 42, 264-268; Picken, R.N., Mazaitis, A.J., Maas, W.K., Rey, M. & Heyneker, H. (1983) Infection & Immunity 42, 269-275) were amplified from the PvuI-linearized pCF2 (expression vector for human IFN-ω1 spliced to the STII leader sequence, total sequence found in file pCF2Seq.DOC) using the 5' (CGTCTTCAAGAATTCGAGATTATCG, EcoRI site underlined) and 3' (GGCAGATCACATGCATAGGCATTTGTAGCAATAG) primers. The purified PCR products were combined and amplified using the 3' primer of the first and the 5' primer of the second PCR reaction. The EcoRI/HindIII-cut

PCR product was cloned into the corresponding sites of Bluescribe M13$^+$; the nature of the insert was verified by sequencing (pBS-STII-IFNα2c).

pDH10, pDH11: The XhoI-HindIII fragment from pBS-STII-IFNα2c was isolated and ligated into XhoI-HindIII doubly restricted pRH284/T (phoA promoter construct, pDH10) or into XhoI-HindIII doubly restricted pRH281/5 (trp promoter construct, pDH11).

Both plasmids were used to transform E.coli HB101 .

Fermentation and Extraction

1. HB101/pDH10 (phoA-promoter)

Medium: Na^+, K^+, NH_4^+, Mg^{++}, Ca^{++}, $SO_4^=$, PO_4 in limiting concentration, Cl^-
vitamins, trace elements,
yeast extract, glucose,

Fermentation parameters: temperature 28°C, pH = 6,5
induction of IFN-α_2 expression takes place by phosphate depletion in the medium because of growth of E. coli

Extraction of biomass: a) 10 minutes in 1% SDS at 70°C in the water bath or
b) High pressure homogenisator at 1600 bar

Yield: 0,1 to 0,2 mg/l.OD or g/kg biomass, resp.

2. HB101/pDH11 (trp-promoter)

Medium: Na^+, K^+, NH_4^+, Mg^{++}, Ca^{++}, $SO_4^=$, PO_4, Cl^-
vitamins, trace elements,
yeast extract, glucose,

Fermentation parameters: Temperatur 28°C, pH = 6,5
induction of IFN-α_2 expression with 3-β-indoleacrylic acid at start of fermentation;

Extraction of biomass: a) 10 minutes in 1% SDS at 70°C in the water bath or
b) High pressure homogenisator at 1600 bar

Yield: ca. 0,06 mg/l.OD or g/kg Biomasse, resp.

ELISA: biomass was diluted and measured in an ELISA against pure IFN-a2c as standard.

Biochem. J. (1994) 298, 719–725 (Printed in Great Britain)

Exhibit

Periplasmic expression of human interferon-α2c in *Escherichia coli* results in a correctly folded molecule

Tilman VOSS,* Edgar FALKNER, Horst AHORN, Edeltraud KRYSTEK, Ingrid MAURER-FOGY, Gerhard BODO and Rudolf HAUPTMANN

Ernst-Boehringer Institut für Arzneimittelforschung, Bender & Co., Dr. Boehringer-Gasse 5–11, A-1121 Vienna, Austria

Human interferon-α2c (IFN-α2c) was produced in *Escherichia coli* under the control of the alkaline phosphatase promoter using a periplasmic expression system. Compared with other leader sequences, the heat-stable entertoxin II leader of *E. coli* (*STII*) resulted in the highest rate of correct processing as judged by Western-blot analysis. The fermentation was designed as a batch-fed process in order to obtain a high yield of biomass. The processing rate of IFN-α2c could be increased from 25% to more than 50% by shifting the fermentation pH from 7.0 to 6.7. IFN-α2c extracted from the periplasm was purified by a new four-step chromatographic procedure. Whereas cytoplasmically produced IFN-α2c does not have its full native structure, IFN-α2c extracted from the periplasm was found to be correctly folded, as shown by c.d. spectroscopy. Peptide-map analysis in combination with m.s. revealed the correct formation of disulphide bridges. N-terminal sequence analysis showed complete removal of the leader sequence, creating the authentic N-terminus starting with cysteine.

INTRODUCTION

Human interferon-α2 (IFN-α2) was among the first proteins to be produced by recombinant DNA technology (Goeddel et al., 1980; Dworkin-Rastl et al., 1983; Thatcher and Panayotatos, 1986). The recombinant host was *Escherichia coli* and the protein was produced by cytoplasmic expression (Bodo and Maurer-Fogy, 1986).

There are, however, several serious drawbacks to the cytoplasmic expression of recombinant proteins in *E. coli*. The intracellularly synthesized protein remains in the reduced state and may either form insoluble inclusion bodies or is found in the soluble fraction after rupture of the bacterial cell wall. This protein does not have the correct conformation, and disulphide bridges are not formed. In order to obtain a native protein, the molecule has to be oxidized and refolded to achieve the correct conformation. This step, however, is not very efficient and in addition generates a variety of unwanted molecular forms that can be detected in such preparations: residual-reduced and partially reduced forms, oligomers formed by intermolecular disulphide bridges and molecular forms with unnatural intramolecular disulphide bridges ('scrambled' forms). The occurrence of these forms has been described for recombinant IFN-α2c (Bodo and Maurer-Fogy, 1986).

The removal of the N-terminal methionine residue which marks the beginning of translation presents another problem of cytoplasmic expression. The cleavage efficiency of the aminopeptidase depends on the subsequent residues. IFN-α2c with an N-terminal cysteine is a rather poor substrate. In addition, the cleavage efficiency is significantly reduced at the high expression rates obtained later in the fermentation process. Therefore fermentation has to be stopped at an early time point, at low cell density and with low IFN-α2c yields, as the methionine-containing protein cannot be separated from the form without methionine. Most of these unwanted molecular forms can be minimized during the purification of the protein by preparative separation techniques, albeit at the price of rather low yields of the correct form of IFN-α2c.

In the present paper we describe an alternative approach to the production of human IFN-α2c in *E. coli*, using the signal peptide from heat-stable enterotoxin II of *E. coli* (*STII*) (Lee et al., 1983; Picken et al., 1983) which leads to the secretion of IFN-α2c into the periplasm. In contrast with cytoplasmic expression, periplasmic secretion has been shown to yield a correctly folded native molecule with correct N-terminal processing and formation of disulphide bridges.

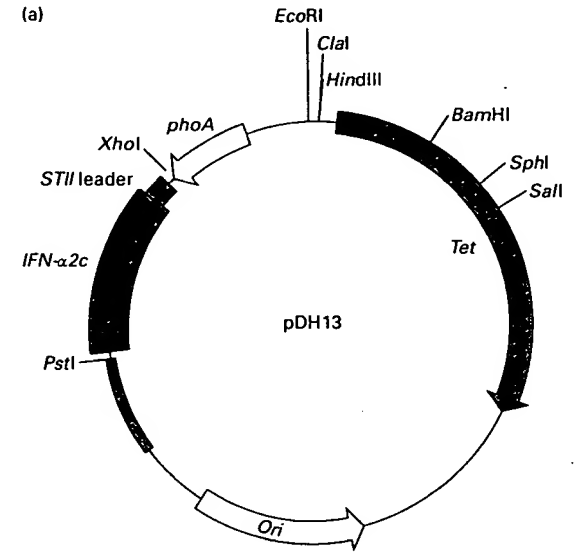
MATERIALS AND METHODS

Construction of the expression vector

All cloning procedures were performed essentially following standard protocols (Sambrook et al., 1989). Construction of the expression cassette containing the phosphatase promoter, the *STII* leader and the human IFN-α2c was performed by splicing by overlap extension (SOE)–PCR (Ho et al., 1989). The IFN-α2c sequence was PCR-amplified from the *Hin*dIII-linearized bacterial expression construct pER21/1 (Dworkin-Rastl et al., 1983) using the 5′ primer (ATGCCTATGCATGTGATCTGCCTCAAACCCACAGC) and the 3′ primer (GACTTCAGAAGCTTCTGCAGTTACGATCGTTATCATTCCTTACTTCTTAAACTTTC, *Hin*dIII site underlined). The phosphatase promoter (Chang et al., 1986; Shuttleworth et al., 1986) and the *STII* leader (Lee et al., 1983; Picken et al., 1983) were amplified from the *Pvu*I-linearized pCF2 (expression vector for human IFN-ω1 spliced to the *STII* leader sequence; R. Hauptmann, unpublished work) using the 5′ (CGTCTTCAAGAATTCGAGATTATCG, *Eco*RI site underlined) and 3′ (GGCAGATCACATGCATAGGCATTTGTAGCAATAG) primers. The purified PCR products were combined and amplified using the 3′ primer of the first and the 5′ primer of the second PCR reaction. The *Eco*RI–*Hin*dIII-cut PCR product was cloned into the corresponding

Abbreviations used: IFN-α, interferon-α; *STII*, heat-stable enterotoxin II leader of *Escherichia coli*; SOE, splicing by overlap extension; RP-h.p.l.c., reversed-phase h.p.l.c.

* To whom correspondence should be addressed.



(b)

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EcoRI
 1 gaattcgagattatcgtcactgcaatgcttcgcaatatggcgcaaaatgaccaacagcggttgattgatcaggtagaggg  80
81 ggcgctgtacgaggtaaagcccgatgccagcattcctgacgacgatacggagctgctgcgcgattacgtaaagaagttat 160
161 tgaagcatcctcgtcagtaaaaagttaatcttttcaacagctgtcataaagttgtcacggccgagacttatagtcgcttt 240
                XhoI                STII leader peptide →
241 gtttttatttttaatgtatttgctcgaaggttgaggtgatttt ATG AAA AAG AAT ATC GCA TTT CTT    309
                                                 m  k  k  n  i  a  f  l
                                                              IFN-α2c →
310 CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA TGT GAT CTG CCT CAA 369
     l  a  s  m  f  v  f  s  i  a  t  n  a  y  a  C  D  L  P  Q   5
370 ACC CAC AGC CTG GGT AGC AGG AGG ACC TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT 429
     T  H  S  L  G  S  R  R  T  L  M  L  L  A  Q  M  R  R  I  S  25
430 CTT TTC TCC TGC TTG AAG GAC AGA CGT GAC TTT GGA TTT CCC CAG GAG GAG TTT GGC AAC 489
     L  F  S  C  L  K  D  R  R  D  F  G  F  P  Q  E  E  F  G  N  45
490 CAG TTC CAA AAG GCT GAA ACC ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC AAT 549
     Q  F  Q  K  A  E  T  I  P  V  L  H  E  M  I  Q  Q  I  F  N  65
550 CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG ACC CTC CTA GAC AAA TTC TAC 609
     L  F  S  T  K  D  S  S  A  A  W  D  E  T  L  L  D  K  F  Y  85
610 ACT GAA CTC TAC CAG CAG CTG AAT GAC CTG GAA GCC TGT GTG ATA CAG GGG GTG GGG GTG 669
     T  E  L  Y  Q  Q  L  N  D  L  E  A  C  V  I  Q  G  V  G  V  105
670 ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG GCT GTG AGG AAA TAC TTC CAA AGA 729
     T  E  T  P  L  M  K  E  D  S  I  L  A  V  R  K  Y  F  Q  R  125
730 ATC ACT CTC TAT CTG AAA GAG AAG AAA TAC AGC CCT TGT GCC TGG GAG GTT GTC AGA GCA 789
     I  T  L  Y  L  K  E  K  K  Y  S  P  C  A  W  E  V  V  R  A  145
790 GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC TTG CAA GAA AGT TTA AGA AGT AAG GAA 849
     E  I  M  R  S  F  S  L  S  T  N  L  Q  E  S  L  R  S  K  E  165
          PvuI  PstI
850 tgataacgatcgtaactgcag
```

Figure 1 (a) Map of the plasmid pDH13 and (b) sequence of the *STII* leader–IFN-α2c fusion construct (*Eco*RI–*Pst*I fragment)

(a) *phoA*, alkaline phosphatase promoter; *Tet*, tetracycline-resistance gene. (b) The *STII* leader sequence is shown in small letters. The underlined sequences show the position of the respective restriction enzyme-cleavage sites.

sites of Bluescribe M13$^+$; the nature of the insert was verified by sequencing.

The final expression plasmid pDH13 was obtained by inserting the expression cassette (*Eco*RI cut, filled in with Klenow, recut with *Pst*I fragment from the Bluescribe intermediate) into the large *Ssp*I–*Pst*I fragment of pAT153 (Twigg and Sherratt, 1980). The correct assembly of the resulting expression plasmid pDH13 (Figure 1a) was verified by restriction enzyme analysis. The sequence of the *STII* leader and IFN-α2c is shown in Figure 1(b).

Construction of expression vectors containing various leader sequences

In order to combine the reading frame for IFN-α2c with the coding sequences of other bacterial and phage leader sequences (Table 1) oligonucleotide pairs with *Eco*RI and *Hin*dIII compatible ends were synthesized (Figure 2) and subcloned into Bluescribe M13$^+$ cleaved with these two enzymes. The inserts were verified by sequencing. These constructs and pDH13 were used as templates for SOE–PCR for splicing the individual leader sequences on to the coding sequence of IFN-α2c. The resulting PCR products were cut with *Eco*RI and *Hin*dIII and subcloned into Bluescribe M13$^+$. After sequence verification, the *Xho*I–*Pst*I fragments were isolated and ligated into the large *Xho*I–*Pst*I fragment of pDH13. The resulting plasmids were used to transform *E. coli* W3110 for the expression of the recombinant proteins.

Fermentation

Preculture

Autoclaved Luria–Bertani medium (700 ml) plus 5 mg/l tetracycline (sterile-filtered) in a 2-litre glass vessel were inoculated from a stock culture to an A_{546} of 0.01. The culture was incubated for 12 h at 37 °C with vigorous stirring (800 rev./min) and aeration (5 fermenter vol./min).

Main culture

Salts [10 mM $(NH_4)_2HPO_4$, 30 mM $(NH_4)_2SO_4$, 40 mM K_2HPO_4, 10 mM NaCl, 4.7 mM NH_4Cl, 3.4 mM trisodium citrate] were heat-sterilized in the fermenter. Trace elements, $MgSO_4$, glucose, thiamine, L-tryptophan, L-leucine, L-methionine and tetracycline, all sterile-filtered presterilized, were added aseptically after cooling, resulting in a starting volume of about 7 litres. Then 600 ml of the preculture was automatically inoculated into the fermenter. Fermentation conditions were: agitation 1000 rev./min, aeration 1 fermenter vol./min, back pressure 0.3×10^5 Pa, temperature 37 ± 0.1 °C; the pH was maintained at 6.7 ± 0.1 with NH_3 and H_2SO_4. The concentration of dissolved oxygen was kept above 15% air saturation (at 0.3×10^5 Pa back pressure) by aeration with oxygen-enriched air as necessary. After the initially available glucose had been consumed, a feed procedure was started, automatically triggered by the oxygen concentration, which contained glucose, thiamine, $MgSO_4$, L-tryptophan, L-leucine and L-methionine. The feeding rate started at 2.5 g of glucose/l per h and was continuously increased for 24 h to 5.0 g/l per h and then kept constant until the end of the fermentation process.

The fermentation run was terminated when a total of 350 g/l glucose had been added. At this time a typical A_{546} of 220–250 was obtained.

The broth was then cooled to about 10 °C and simultaneously adjusted to pH 2.0 with H_2SO_4 for inactivation of the biomass. The biomass was harvested by centrifugation and stored frozen at −70 °C.

Extraction and purification

Acid-inactivated biomass (approx. 0.5 kg) was suspended in

Table 1 DNA and amino acid sequence of the different signal sequences used for secretion of IFN-α2c into the periplasm of *E. coli*

M13, M13 procoat protein. The codon of the first residue of the *phoA* leader peptide was changed from GTG (valine) to ATG (methionine).

Gene bank accession no.	Leader	DNA and protein sequence
	STII	ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA
		M K K N I A F L L A S M F V F S I A T N A Y A
M13763	*phoA*	ATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC
		M K Q A T I A L A L L P L L F T P V T K A
J01655	*ompF*	ATG ATG AAG CGC AAT ATT CGT GCA GTG ATC GTC CCT GCT CTG TTA GTA GCA GGT ACT GCA AAC GCT
		M M K R N I L A V I V P A L L V A G T A N A
M16643	*lamB*	ATG ATG ATT ACT CTG GCG AAA CTT CCT CTG GCG GTT GCC GTC GCA GCG GGC GTA ATG TCT GCT GAG GCA ATG GCT
		M M I T L R K L P L A V V V A A G V M S A Q A M A
V00303	*malE*	ATG AAA ATA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC
		M K I T G A R I L A L S A L T T M M F S A S A L A
V00307	*ompA*	ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC
		M K K T A I A I A V A L A G F A T V A Q A
J01645	*lpp*	ATG AAA GCT ACT AAA CTG GTA CTG GGC GCG GTA ATC CTG GGT TCT ACT CTG CTG GCA GGT
		M K A T K L V L G A V I L G S T L L A A
M11454	*M13*	ATG AAA AAG TCT TTA GTC CTC AAA GCC TCT GTA GCC GTT GCT ACC CTC GTT CCG ATG CGT TCT TTC GCT
		M K K S Y V L K A S V A V A T L V R M L S F A

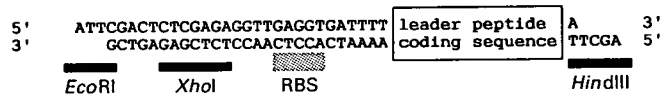

Figure 2 Structure of the synthetic DNA cloned into Bluescribe M13⁺

■, Location of restriction sites; ▨, ribosome-binding site (RBS).

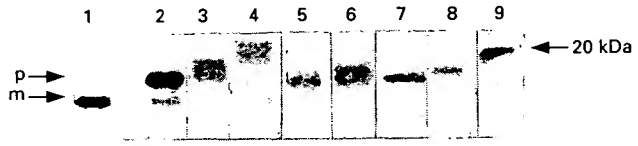

Figure 3 Comparison of the processing efficiency of different leader sequences (Western blot)

IFN-α2c was expressed with different leader sequences in the *E. coli* strain W3110. Lane 1, purified IFN-α2c; lane 2, *STII*; lane 3, *ompF*; lane 4, *M13* precoat protein; lane 5, *malE*; lane 6, *lamB*; lane 7, *ompA*; lane 8, *lpp*; lane 9, *phoA*. m, the position of the processed IFN-α2c; p, position of the unprocessed IFN-α2c, carrying the *STII* leader.

500 ml of 1% acetic acid using a Polytron homogenizer, and stirred at 0 °C for 1 h. Poly(ethylenimine) (50% stock solution; Serva, Heidelberg, Germany) was added to a final concentration of 0.25% (w/v). The suspension was adjusted to a pH of 10.0 with 5 M NaOH and stirred for an additional 2 h at 0 °C. After adjustment of the pH to 7.5 with 5 M HCl, the bacteria were pelleted by centrifugation at 17000 *g* (Beckman J2-21 centrifuge). The supernatant was loaded on to a silica column (35 mg of protein/ml of column material) equilibrated in 20 mM Tris/HCl, pH 7.5. The column was washed with 30 column vol. of this buffer, followed by a wash step with 20 mM Tris/HCl, pH 7.5, containing 100 mM tetramethylammonium chloride. IFN-α2c was eluted by increasing the tetramethylammonium chloride concentration to 800 mM.

The IFN-α2c-containing fractions were pooled, adjusted to 20% $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and loaded on to a phenyl-Sepharose column (Phenyl Toyopearl 650 S; Tosohaas) equilibrated in 20 mM Tris/HCl, pH 7.5, containing 30% $(NH_4)_2SO_4$ (loading conditions). IFN-α2c was eluted from the column with a linear gradient from 100% loading conditions to 100% 20 mM Tris/HCl, pH 7.5, containing 30% ethylene glycol (solvent B). The pooled IFN-α2c-containing fractions were adjusted to 20 mM sodium succinate, pH 5.0, by extensive dialysis. The final pH was adjusted to 3.0 with HCl before loading on to sulphopropyl ion-exchange resin (Toyopearl TSK SP 5PW; Tosohaas) equilibrated in 20 mM sodium succinate, pH 5.0 (loading conditions). IFN-α2c was eluted from the column with a linear gradient from 100% loading conditions to 100% solvent C (20 mM sodium succinate, 500 mM NaCl, 10% ethylene glycol, pH 5.5). The pooled IFN-α2c -containing fractions were dialysed against 10 mM Bistris, pH 5.8, and loaded on to a DEAE-Sepharose (DEAE-Sepharose Fast-Flow; Pharmacia) column equilibrated in the same buffer. Elution of IFN-α2c occurred by a linear gradient into 10 mM BisTris, pH 5.8, containing 500 mM NaCl and 0.1% Tween 20 (solvent D).

Immunoaffinity purification

IFN-α2c expressed either peri- or cyto-plasmically was extracted from the biomass with 1% acetic acid as described above. This extract was neutralized, dialysed against 25 mM Tris/HCl, pH 7.5, and immunoaffinity-purified using the IFN-α2c-specific monoclonal antibody EBI-1 (Adolf et al., 1982) coupled to CNBr-activated Sepharose (Pharmacia), equilibrated with 25 mM Tris/HCl, pH 7.5. IFN-α2c was eluted with 0.1 M citric acid/30% ethylene glycol and dialysed against PBS.

Western blots

Portions of biomass were dissolved in SDS/PAGE loading buffer containing 10 mM dithiothreitol (about 1 mg of total protein/ml) and boiled for 5 min. Samples (15 μl) were separated by SDS/PAGE (15% gels). Western blots were carried out

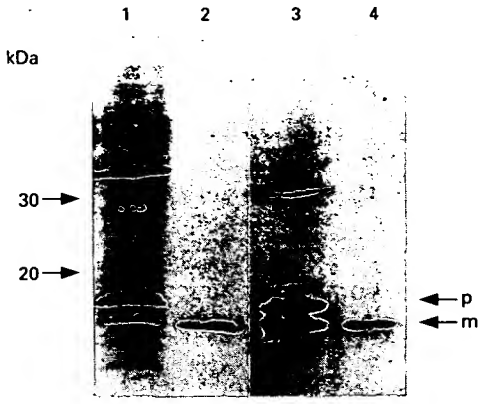

Figure 4 IFN-α2 Western blot of biomass obtained from fermentations at pH 7.0 and pH 6.7

Lane 1, typical fermentation at pH 7.0; lane 3, fermentation at pH 6.7; lanes 2 and 4, purified IFN-α2c. p, unprocessed precursor; m, the mature processed IFN-α2c.

following standard protocols using the IFN-α2c-specific monoclonal antibody EBI-1 (Adolf et al., 1982).

Reversed-phase (RP-) h.p.l.c.

Intact IFN-α2c was analysed on a BakerBond C18 column (4.5 mm × 250 mm, 5 μm particles, 30 nm pore size) at 30 °C. The samples were chromatographed using solvents A (0.1 % trifluoroacetic acid in water) and E (0.1 % trifluoroacetic acid in acetonitrile) using gradient conditions as described in the legend to Figure 7.

Tryptic digest

IFN-α2c was digested with 1 % (w/w) trypsin (sequencing grade, Boehringer-Mannheim) in 1 % NH_4HCO_3. Reduction was performed by incubating the cleavage mixture in 3 M urea/50 mM dithiothreitol for 2 h at 25 °C. RP-h.p.l.c. of the peptides was performed on a DeltaPAK C18-column (Millipore–Waters; 3.9 mm × 150 mm, 5 μm particles, 10 nm pore size) at 30 °C, using the solvents described above. Peptides were eluted using a linear gradient from 0 to 55 % solvent E in 55 min at a flow rate of 1 ml/min.

^{252}Cf plasma-desorption m.s.

Mass spectra of tryptic peptides were obtained on a BIO-ION 20 time-of-flight mass spectrometer (BIO-ION; Nordic AB, Uppsala, Sweden). Samples were dissolved in 0.1 % trifluoroacetic acid/water and applied to nitrocellulose-coated targets (BIO-ION). Spectra were obtained at an acceleration voltage of 17 kV.

C.d. spectroscopy

Spectra of IFN-α2c were measured with a JASCO 600 spectrometer with 10 mm pathlength at 25 °C, and scanned at 0.2 nm intervals at 50 nm/min. IFN-α2c was dissolved at a concentration of 40 μg/ml in 20 mM phosphate buffer, pH 7.4. The concentration was determined by measuring A_{280}. The absorption coefficient was determined using an IFN-α2c standard quantified by amino acid analysis (results not shown).

RESULTS

Selection of the optimal signal sequence

It was thought that the presence of cysteine as the N-terminal amino acid residue of IFN-α2c might affect the efficiency of the enzymic removal of the signal sequence for periplasmic expression. Therefore several different leader sequences from various secreted proteins were tested in small-scale fermentation experiments (10 litres). These sequences are listed in Table 1. Western blots were performed to analyse the processing efficiency which is reflected in the amount of IFN-α2c lacking the signal sequence (Figure 3, band marked m). The signal sequence of *STII* was the only one which revealed partial processing resulting in the expected N-terminus of IFN-α2c (as shown by sequence analysis of the purified protein; results not shown). The processing rate varied between 10 and 20 %. Some of the other sequences examined exhibited unexpectedly high mobilities (Figure 3, lanes 4 and 9), but none showed any reasonable processing.

The processing efficiency of the *STII* leader construct could not be improved by using other *E. coli* strains nor by reducing the fermentation temperature to 32 °C (results not shown).

On the basis of these experiments, an expression system using the *STII* signal sequence and the *E. coli* strain W3110 was chosen for the production of IFN-α2c (Figure 1).

Fermentation

The fermentation process was designed as a batch-fed procedure in order to achieve high cell densities. An A_{546} of 220–250 was reached routinely, resulting in about 1.8 kg of biomass/10 l fermenter volume. The expression of IFN-α2c driven by the promoter of the alkaline phosphatase (*phoA*) was induced by phosphate concentration limitation in the medium. The average yield was routinely about 250–300 mg/l IFN-α2c. Feeding of leucine and methionine was found to be necessary in order to inhibit the incorporation of norleucine instead of methionine into IFN-α2c.

About 30 % of the IFN-α2 produced was correctly processed by the signal peptidase, as indicated by the ratio of the two IFN-α2c bands in the Western blot (Figure 4). Lowering of the pH of the fermentation broth to 6.7 increased the processing grade to about 50–60 % (Figure 4, lanes 3 and 4). The harvested biomass had an average content of about 1.5 mg of IFN-α2c/g of biomass.

Purification

IFN-α2c secreted into the periplasmic space was extended at low pH followed by a high-pH step. This second step was empirically shown to give more reproducible yields of IFN-α2c (results not shown). The extraction yield of 30–50 % of total IFN-α2c found in the biomass (as determined by e.l.i.s.a.) reflects the amount of the correctly processed IFN-α2c.

IFN-α2c was purified through a series of four chromatographic steps including adsorption chromatography on silica, hydrophobic interaction chromatography, cation- and anion-exchange chromatography. Figure 5 shows a representative set of chromatograms, and Table 2 summarizes the yield of the individual steps. The total yield of the purification procedure is about 14 %. Extraction of 1 kg of biomass with 1.5 mg/g IFN-α2c finally results in about 190 mg of IFN-α2c. Figure 6 shows a representative SDS/PAGE of the individual steps. The final product

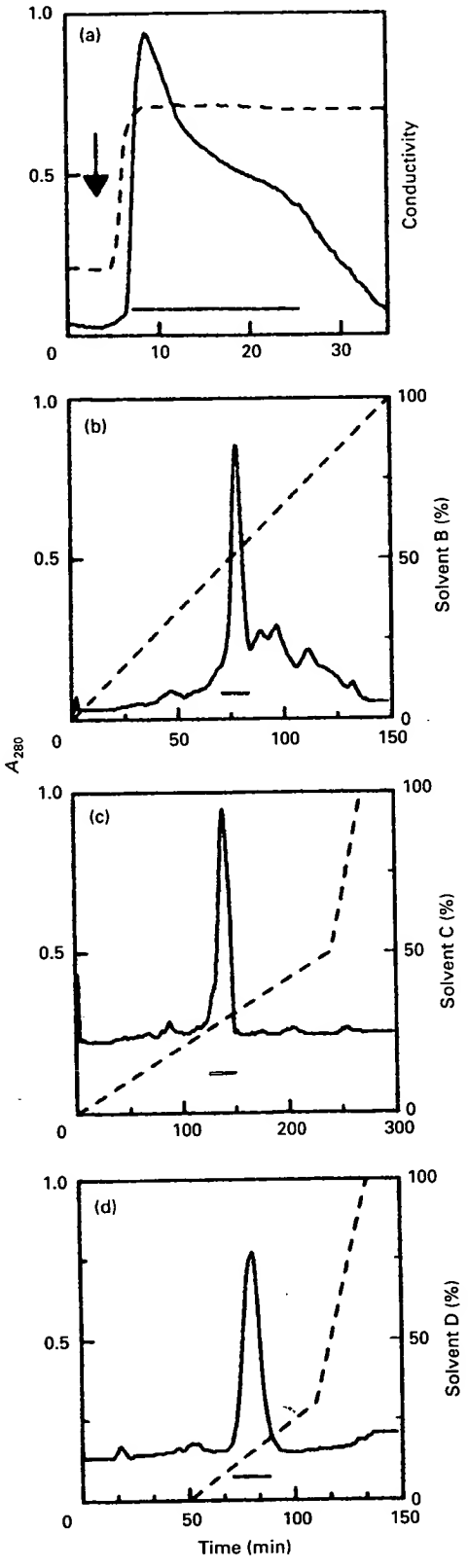

Figure 5 Chromatographic purification of IFN-α2c extracted from biomass

(a) Adsorption chromatography on silica. The arrow indicates the elution with 800 mM tetramethylammonium chloride. ——, Conductivity. (b) Hydrophobic interaction chromatography on phenyl-Sepharose. The elution was performed with a linear gradient from 0 to 100% solvent B as indicated (– – –). (c) TSK sulphopropyl cation-exchange chromatography. The elution was performed with a gradient from 0 to 100% solvent C as indicated (– – –). (d) Anion-exchange chromatography on DEAE-Sepharose. The elution was performed with a gradient from 0 to 100% solvent D as indicated (– – –). The bar beneath the major peaks in each chromatogram indicate the IFN-α2c-containing pools which were collected and used for the following step.

Table 2 Yields of the four-step purification of IFN-α2c

The IFN-α2c content is given as percentage of the total protein content. The recovery is expressed as a percentage of all IFN-α2c loaded on to the respective column.

Step	IFN-α2c content (%)	Recovery (%)
Extraction	4.5	29.3
Silica	16.7	83.4
Phenyl-Sepharose	71.2	93.2
Sulphopropyl resin	97.6	70.9
DEAE-Sepharose	100.0	86.9

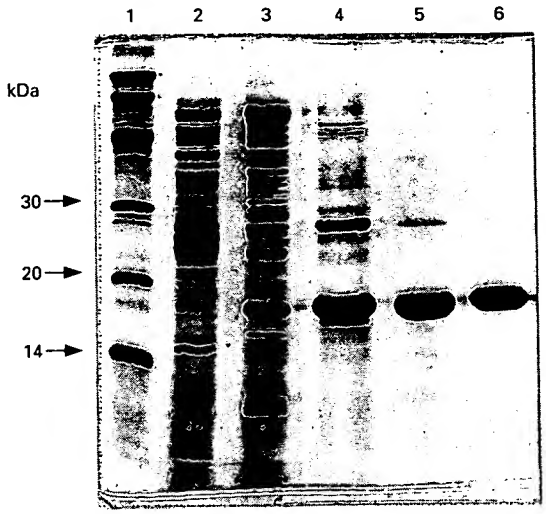

Figure 6 SDS/PAGE of the purification-step products

Lane 1, molecular-mass standards; lane 2, acidic extract; lane 3, silica pool; lane 4, phenyl-Sepharose pool; lane 5, TSK pool; lane 6, DEAE-Sepharose pool. The gel was stained with Coomassie Blue.

(lane 6) is essentially pure. No contaminating bands are observed even on silver staining (results not shown).

Characterization of purified IFN-α2c

In order to demonstrate the correct folding and disulphide-bridge formation of IFN-α2c in the periplasm, the purified protein was analysed by RP-h.p.l.c., by tryptic peptide maps and by c.d. spectroscopy. For this purpose, IFN-α2c was extracted as described and rapidly purified by immunoaffinity chromatography. Significant disulphide formation or protein folding is minimal under these conditions. For comparison, cytoplasmically expressed IFN-α2c (expression system without any leader sequence) was extracted and purified similarly. These preparations have been shown previously to contain reduced and 'scrambled' forms (Bodo and Maurer-Fogy, 1986).

The periplasmically expressed IFN-α2c exhibited a single sharp peak in the RP-h.p.l.c. profile (Figure 7, tracing 1). No scrambled

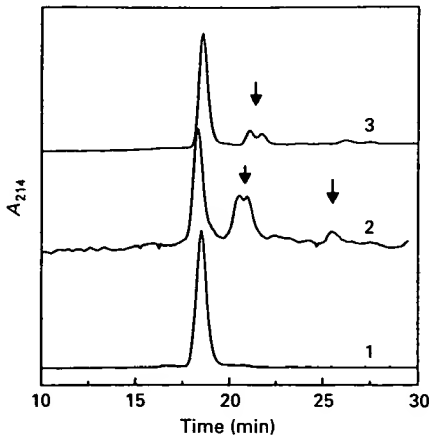

Figure 7 RP-h.p.l.c. profiles of immunoaffinity-purified IFN-α2c obtained from periplasmic secretion (1) compared with IFN-α2c from cytoplasmic expression before (2) and after (3) oxidation

The arrows indicate various IFN-α2c species with incorrectly assembled disulphide bonds obtained from cytoplasmic expression. Elution was performed with a linear gradient (flow rate 1 ml/min) from 45 to 53% solvent E.

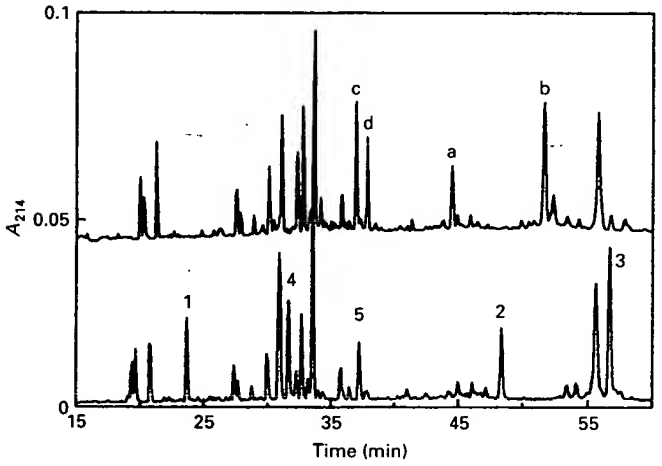

Figure 8 Tryptic peptide map of IFN-α2c before (upper tracing) and after (lower tracing) reduction with dithiothreitol

a, Disulphide-linked peptides 1–12 + 84–112; b, disulphide-linked peptides 1–12 + 71–112; c, disulphide-linked peptides 24–31 + 134–144; d, disulphide-linked peptides 24–31 + 135–144. 1, Peptide 1–12; 2, peptide 84–112; 3, peptide 71–112; 4, peptide 134/135–144; 5, peptide 24–31. The peptides marked a, c and b, d were isolated and subjected to m.s. (Table 3). The identity of peptides 1–5 was verified by N-terminal sequencing (results not shown).

Table 3 Plasma-desorption analysis of disulphide-linked tryptic peptides

The numbers indicate the positions of the peptides in the sequence. a, b, c and d refer to the positions of the respective peptides in the tryptic map (Figure 8).

Peptide	Calculated mass (Da)	Observed mass (Da)
1–12 + 84–112 (a)	4615.2	4618.3
1–12 + 71–112 (b)	6047.8	6049.0
24–31 + 134–144 (c)	2247.6	2246.4
24–31 + 135–144 (d)	2119.4	2119.9

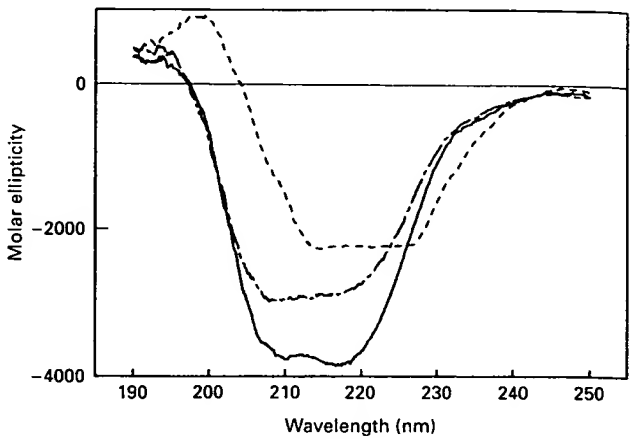

Figure 9 Comparison of c.d. spectra of immunoaffinity-purified IFN-α2c obtained from periplasmic secretion and cytoplasmic expression

——, Periplasmic secretion; – – –, cytoplasmic expression before oxidation; –·–, cytoplasmic expression after oxidation. Molar ellipticity is expressed as $[\theta] \times 10^{-3}$ degree·cm^2·dmol^{-1} using a mean residual weight of 116.87.

forms were seen. These forms account for up to 50% of the cytoplasmically expressed IFN-α2c (Figure 7, tracing 2, arrows). Reoxidation can significantly reduce the amount of these forms (Figure 7, tracing 3).

In addition, the correct alignment of the two disulphide bridges in the periplasmically expressed IFN-α2c was demonstrated by analysis of tryptic peptide maps of the reduced and unreduced protein (Figure 8). The dithiothreitol-sensitive peaks were shown by plasma-desorption m.s. to contain the expected disulphide-linked peptides 1–12 + 71/84–112 (a, b) and 24–31 + 134/135–144 (c, d). The m.s. data of these four peptides which are generated by the alternative use of tryptic-cleavage sites are summarized in Table 3. The identity of the reduced peptides (1–5, Figure 8) was verified by N-terminal sequence analysis (results not shown).

The correct folding of secreted IFN-α2c was demonstrated by c.d. spectroscopy and compared with cytoplasmically expressed IFN-α2c (Figure 9). Periplasmic IFN-α2c exhibited the typical appearance expected for a mostly α-helical type-I IFN (Senda et al., 1992), which is characterized by the pronounced negative peaks around 210 and 218 nm. The spectrum of cytoplasmically expressed IFN-α2c (Figure 9) was noticeably different. The characteristic peaks around 215 nm were slightly shifted to longer wavelengths and were less intense. An additional positive peak was observed at 200 nm, and this altogether indicates a reduced amount of secondary-structure elements. The spectrum of reoxidized material (Figure 9) showed a greater resemblance to the spectrum of the periplasmically expressed material, although the intensity of the correctly located negative peaks was slightly reduced.

DISCUSSION

High-level expression of eukaryotic proteins in *E. coli* frequently results in the formation of insoluble aggregates, known as the inclusion bodies (Schein, 1989), or in incorrectly processed and misfolded proteins, sometimes with incorrect disulphide-bridge formation.

The data presented here demonstrate that such errors, which are observed in cytoplasmically expressed IFN-α2c, are overcome by periplasmic secretion. A more detailed analysis showed that not every signal sequence tested resulted in the expected pro-

cessing. Furthermore, significant differences in the processing efficiency were observed between different *E. coli* strains (results not shown). The combination of the heat-stable enterotoxin signal sequence (*STII*) and strain W3110 gave the best results. However, the processing was never quantitative, leaving 50–80% of the precursor unaffected. The yield of correctly processed IFN-α2c could be improved to over 50% by lowering the pH in the fermenter from 7.0 to 6.7. Fermentation at lower temperatures, which might enhance the solubility of recombinant proteins (Schein, 1989) and therefore their availability for secretion and processing, did not exhibit any beneficial effects (results not shown). The reasons for the incomplete processing remain unclear. Possibly, the high expression rate of the recombinant protein exceeds the capabilities of the secretory pathway. Attempts to overexpress either a cytoplasmic component of this pathway, the chaperone SecB, or the signal peptidase (R. Hauptmann, unpublished work) did not improve the processing rate.

The fermentation process itself was designed as a batch-fed procedure, thus resulting in a high yield of biomass. The expression of IFN-α2c under the control of the alkaline phosphatase promoter was induced by phosphate limitation in the medium. Using this promoter, the onset of the expression of the recombinant protein can be easily controlled. Total yields of about 250–300 mg of IFN-α2c/l with and without signal peptide were routinely obtained at the 10- and 100-litre fermenter scale.

Processing of the precursor yields some 30–50% mature IFN-α2c and this was virtually all recovered by the extraction procedure. The precursor carrying the signal peptide was never observed in the extracts. Purified IFN-α2c contained the correct N-terminal sequence starting with cysteine.

IFN-α2c was extracted rapidly and purified by immunoaffinity chromatography in order to reduce refolding and was shown to be correctly processed and folded. The c.d. spectra exhibited typical α-helical features with a pronounced minimum around 215 nm. The correct formation of the two disulphide bridges between Cys-1 and Cys-98, and Cys-29 and Cys-138, was shown by tryptic peptide mapping in combination with m.s. By contrast, the c.d. spectra of cytoplasmically expressed IFN-α2c looked quite different: after rapid extraction and immunoaffinity purification which reduces refolding, RP-h.p.l.c. revealed that the material contained up to 50% scrambled forms (Bodo and Maurer-Fogy, 1986) with incorrect or open disulphide bridges. The c.d. spectrum of this material was shifted to higher wavelengths, and the bands were less intense. The spectrum of refolded material containing fewer scrambled forms had the appearance of the correctly folded material, but the intensity of the bands was lower. Chromatographic removal of the scrambled forms (Bodo and Maurer-Fogy, 1986) resulted in material that exhibited a c.d. spectrum identical with that of the periplasmically expressed IFN-α2c (results not shown).

All these data indicate that periplasmically expressed IFN-α2c is produced as a correctly folded molecule. This is further substantiated by the full biological activity of 2.2×10^8 units/mg in an antiviral assay (Adolf, 1987), which is identical with the values obtained for natural human IFN-α2 (Adolf et al., 1991).

A new purification procedure was developed for IFN-α2c secreted into the periplasmic space of *E. coli*. This purification protocol included four chromatographic steps, circumventing the problems associated with the use of immunoaffinity chromatography. The yields of the individual steps were usually above 70%, thus resulting in a yield of 40% for the chromatographic purification and 14% for the overall procedure including the extraction step.

In conclusion, the periplasmic expression system described in this study combined with a new efficient purification procedure permits the production of recombinant human IFN-α2c suitable for therapeutic purposes without encountering the problems observed with cytoplasmic expression, such as incomplete removal of the N-terminal methionine or misfolding and incorrect disulphide-bridge formation.

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